REVIEW

Histone Deacetylase Inhibitors: Inducers of Differentiation or Apoptosis of Transformed Cells

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Histone deacetylase (HDAC) inhibitors have been shown to be potent inducers of growth arrest, differentiation, and/or apoptotic cell death of transformed cells *in vitro* and *in vivo*. One class of HDAC inhibitors, hydroxamic acid-based hybrid polar compounds (HPCs), induce differentiation at micromolar or lower concentrations. Studies (x-ray crystallographic) showed that the catalytic site of HDAC has a tubular structure with a zinc atom at its base and that these HDAC inhibitors, such as suberoylanilide hydroxamic acid and trichostatin A, fit into this structure with the hydroxamic moiety of the inhibitor binding to the zinc. HDAC inhibitors cause acetylated histones to accumulate in both tumor and normal tissues, and this accumulation can be used as a marker of the biologic activity of the HDAC inhibitors. Hydroxamic acid-based HPCs act selectively to inhibit tumor cell growth at levels that have little or no toxicity for normal cells. These compounds also act selectively on gene expression, altering the expression of only about 2% of the genes expressed in cultured tumor cells. In general, chromatin fractions enriched in actively transcribed genes are also enriched in highly acetylated core histones, whereas silent genes are associated with nucleosomes with a low level of acetylation. However, HDACs can also acetylate proteins other than histones in nucleosomes. The role that these other targets play in the induction of cell growth arrest, differentiation, and/or apoptotic cell death has not been determined. Our working hypothesis is that inhibition of HDAC activity leads to the modulation of expression of a specific set of genes that, in turn, result in growth arrest, differentiation, and/or apoptotic cell death. The hydroxamic acid-based HPCs are potentially effective agents for cancer therapy and, possibly, cancer chemoprevention. [J Natl Cancer Inst 2000;92:1210–6]

Neoplastic transformation is characterized by inappropriate cell proliferation and/or altered patterns of cell death. However, neoplastic transformation does not necessarily destroy the potential for expression of differentiated characteristics, including cessation of proliferation under appropriate environmental conditions *(1)*. For example, cells infected with temperature-sensitive transforming viruses *(2)* can display either normal or transformed properties, depending on the activity of a temperature-sensitive viral protein. Some malignant cells (e.g., from teratocarcinomas, neuroblastomas, or leukemias) can differentiate along apparently normal pathways when placed in a normal embryonic environment *(3–7)*. In addition, various chemical agents [hybrid polar compounds (HPCs) *(8–10)*, retinoids *(11–15)*, vitamin D_3 *(16)*, and several other agents *(17–19)*] can induce certain transformed cells *in vitro* to express differentiated characteristics and stop proliferating.

Histones are part of the core proteins of nucleosomes. Acetylation and deacetylation of these proteins play a role in the regulation of gene expression *(20)*. There are two classes of enzymes involved in determining the state of acetylation of histones, histone acetyl transferases (HATs) and histone deacetylases (HDACs). There are several reports *(21–24)* that altered HAT or HDAC activity is associated with cancers.

During the last decade, a number of HDAC inhibitors have been identified that induce cultured tumor cells to undergo growth arrest, differentiation, and/or apoptotic cell death *(25–35)*. These agents also inhibit the growth of cancer cells in animal models *(32,35–40)*, and several agents, in particular, hydroxamic acid-based HDAC inhibitors, inhibit tumor growth in animals at doses that are apparently nontoxic and appear to be selective.

This review focuses on studies of HDAC inhibitors, especially on the hydroxamic acid-based HPCs. These compounds represent a class of agents that are potentially effective cancer therapies. (Studies were identified for this review by searching the MEDLINE® database for appropriate papers published in the last 10 years and by a review of bibliographies from articles identified through that search. In addition, we include some of our unpublished data.)

Histone Acetylation and Deacetylation and Gene Expression

Structure of Nucleosomes

Analyses (x-ray and electron crystallographic) show that nucleosomes contain an average of 150 base pairs of DNA wrapped around the nucleosomal core of histones in 1.75 turns of left-handed superhelical DNA *(41–43)*. Five classes of histones have been identified in chromatin: histones H1, H2A, H2B, H3, and H4. Each nucleosome contains two H2As, two H2Bs, two H3s, and two H4s in the core (Fig. 1). Histone H1 occurs in chromatin in about half the amount of the other types of histones and appears to lie on the outer portion of the nucleosome.

Role of Histone Acetylases and Deacetylases

Histones of the nucleosomal core can be acetylated and deacetylated, and the amount of acetylation is controlled by the opposing activities of two types of enzymes, HATs and HDACs. Substrates for these enzymes include ε-amino groups of lysine

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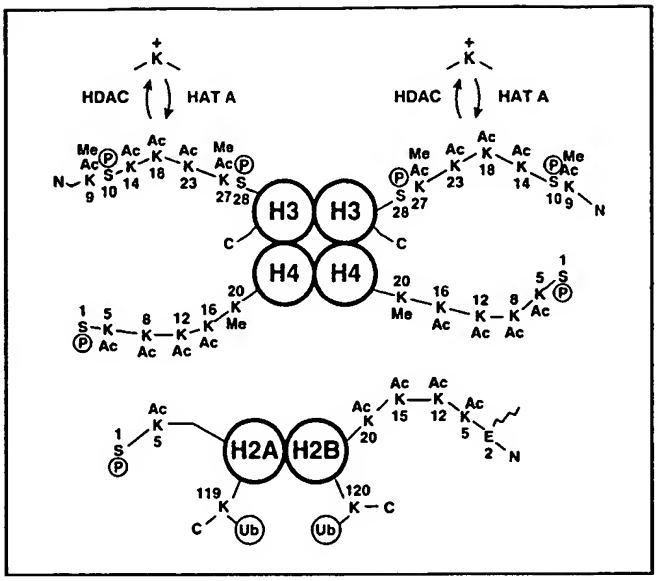


Fig. 1. Histones in nucleosomes. Lysines (K) in the amino (N)-terminal tails of histones H3, H4, H2A, and H2B are potential acetylation/deacetylation sites for histone acetyltransferase (HAT) and histone deacetylase (HDAC). K^+ = positively charged lysine, Ub = ubiquitin, P = phosphate, Ac = acetyl, S = serine, E = glutamic acid, and Me = methyl. [Adapted with permission from Davie *(44).*]

residues located in the amino-terminal tails of the histones. When HDAC removes the acetyl group from histone lysine, it restores a positive charge to the lysine residue condensing the structure of nucleosomes *(44).*

HATs and HDACs

There are at least four groups of proteins with intrinsic HAT activity *(45–50).* The first group contains the GCN5 and P/CAF proteins, which are related to yeast HAT GCN5. The second group contains the closely related cyclic adenosine monophosphate response element-binding protein (CBP) and p300, which act as coactivators for a number of transcription factor complexes. The third group contains the TAF250 protein, part of the basic transcription complex TFIID that binds the TATA box. The fourth group contains the SRC-1 and ACTR proteins that are coactivators for ligand-activated nuclear receptors. In addition, there are probably several other proteins with HAT activity, such as BRCA2, that are part of transcription complexes. HATs play a role in activation of gene expression and may also be involved in gene repression, as suggested by the observation in *Drosophila* that acetylation of the transcription factor T-cell factor by CBP represses transcription *(51).*

Eight HDACs have been described in mammalian cells *(45,52–59).* The yeast RPD3 homologues are HDAC1, HDAC2, HDAC3, and HDAC8; the yeast HDA1 homologues are HDAC4 (also known as HDAC-A), HDAC5 (also known as mHDA1), HDAC6 (also known as mHDAC2), and HDAC7.

Regulation of Transcription

Chromatin fractions enriched in actively transcribed genes are also enriched in highly acetylated core histones *(20,42,45),* whereas silent genes are associated with nucleosomes with a low level of acetylation. Allfrey *(60)* first suggested that histone acetylation was involved in the regulation of transcription. During the past decade, considerable evidence has accumulated to establish the role of acetylation and deacetylation of histones in the regulation of transcription *(20,41–43,45).* The following model describes a role for histone acetylation in regulating gene transcription. Nucleosomes containing highly charged hypoacetylated histones bind tightly to the phosphate backbone of DNA, inhibiting transcription, presumably, because transcription factors, regulatory complexes, and RNA polymerase do not have access to the DNA. Acetylation neutralizes the charge of the histones and generates a more open DNA conformation. Transcription factors and the transcription apparatus then have access to the DNA, and expression of the corresponding genes is promoted (Fig. 2).

In addition to HDACs and HATs, other factors are involved in the regulation of chromatin structure, including methyl-CpG-binding protein *(61–63)* and adenosine triphosphate (ATP)-dependent chromatin-remodeling complexes *(64).* These chromatin-modifying complexes interact with HAT and HDAC complexes to regulate transcriptional activity of genes [for a recent review of chromatin methylation, *see (63);* for reviews of the ATP-dependent chromating remodeling complexes, *see (64,65).*]

HDACs are bound to large protein complexes that regulate gene transcription. Mammalian HDAC1 and HDAC2 are associated with the Sin3 complex that includes NCo-R, SMRT, and several other, as yet, unidentified proteins and appear to repress gene expression by deacetylating core histones. In addition to deacetylation of histones, HDACs may also regulate gene expression by deacetylating transcription factors, such as p53, GATA-1, TFIIE, and TFIIF *(66–68).* HDACs may also participate in cell cycle regulation. The transcription repression mediated by RB binding to the transcription factor E2F involves recruitment of HDAC1 or HDAC2 by RB *(69,70).*

Disruption of HAT and/or HDAC Activity and Development of Cancer

Mutations in the CBP gene, which encodes an HAT, are associated with leukemogenesis and the developmental disorder

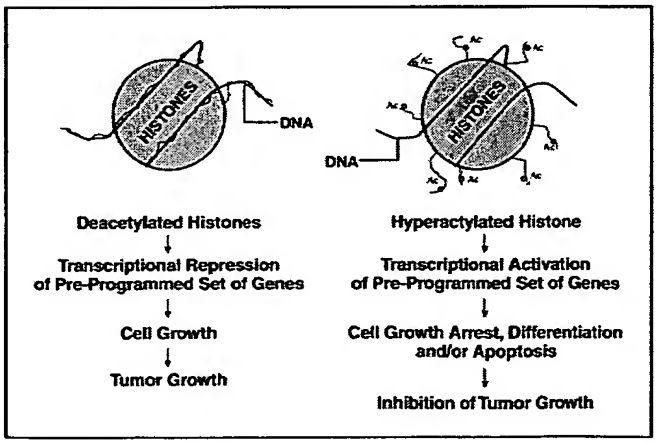


Fig. 2. Proposed mechanism of action of histone deacetylase (HDAC) inhibitors that induce tumor growth arrest, differentiation, and/or apoptotic cell death. With inhibition of HDAC, histones are acetylated (Ac), and the DNA that is tightly wrapped around a deacetylated histone core relaxes. We propose that the accumulation of acetylated histones in nucleosomes leads to expression of specific genes, which, in turn, lead to cell growth arrest, differentiation, and/or apoptotic cell death and, as a consequence, inhibition of tumor growth.

Rubinstein–Taybi syndrome *(71)*. Patients with Rubinstein–Taybi syndrome have a propensity to develop cancer. Microdeletions, translocations, inversions, and various point mutations in the CBP gene have been identified in patients with Rubinstein–Taybi syndrome as well as in patients with some types of colorectal or gastric carcinomas *(21)*. Gene fusions with CBP are associated with several leukemias. In therapy-related acute myeloid and lymphoid leukemias and in myelodysplasia, the CBP gene has been found fused to the MLL gene, and the CBP gene has been found fused in acute myeloid leukemia to the MOZ gene *(72,73)*.

Several leukemogenic transcription factors repress expression of specific genes because of aberrant recruitment of HDACs. This repression of gene expression appears to be an important step in the leukemogenic action of these transcription factors. For example, aberrant recruitment of HDAC activity has been reported in cell lines derived from patients with acute promyelocytic leukemia (APL) *(22–24)*. The oncoprotein encoded by the translocation-generated fusion gene in APL (promyelocytic leukemia [PML]–retinoic acid receptor-α) represses transcription by recruitment of HDAC1. Furthermore, resistance to the differentiating actions of all-*trans*-retinoic acid in a patient with APL was overcome by cotreatment with an inhibitor of HDAC *(74)*. [In a further study *(75)*, four other patients with APL failed to respond.] HDAC-dependent aberrant transcriptional repression is implicated as the main oncogenic mechanism in specific types of myeloid leukemia and lymphoma. For example, in non-Hodgkin's lymphoma, the transcriptional repressor BCL6 is inappropriately overexpressed within the lymphoid compartment, resulting in aberrant transcriptional repression and lymphoid oncogenic transformation *(76)*. Another example is acute myelogenous leukemia of the M2 subtype associated with the t(8;21) chromosomal translocation involving the AML1 and ETO genes *(77)*. The AML1–ETO fusion protein, unlike the AML-1 protein (a transcriptional activator), is a potent dominant transcriptional repressor. In both of these cases, transcriptional repression appears to be mediated by recruitment of HDAC to the transcriptional repressor complex.

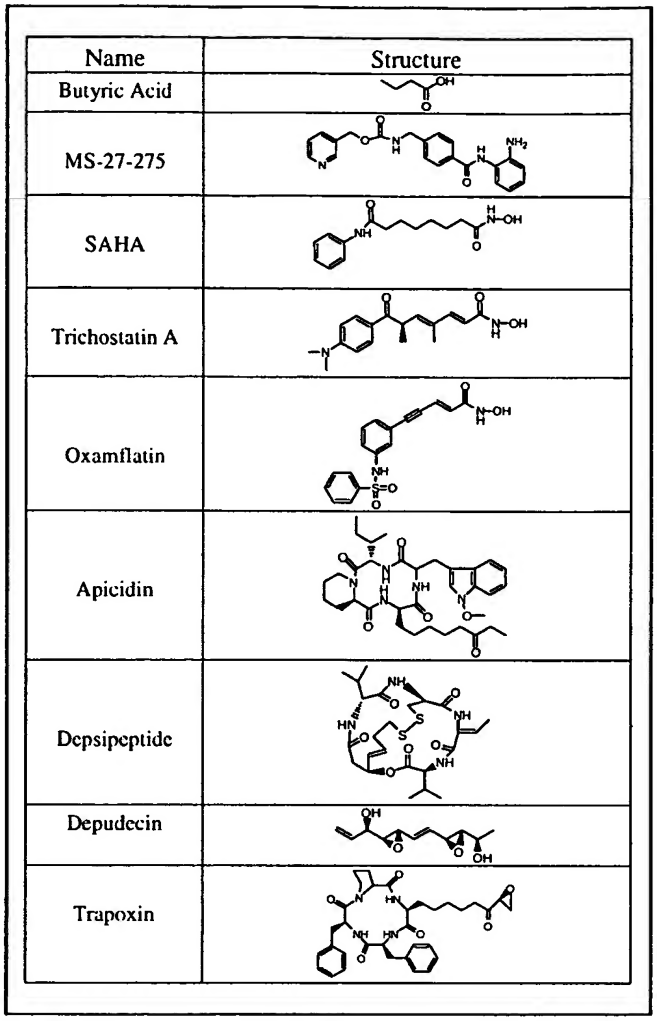
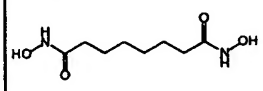
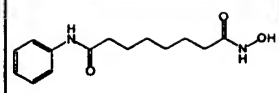
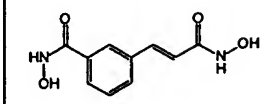
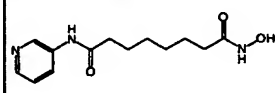


Fig. 3. Histone deacetylase inhibitors (*see* text for references to these inhibitors). SAHA = suberoylanilide hydroxamic acid.

HDAC Inhibitors

Compounds that inhibit HDAC activity are shown in Fig. 3. Several structural classes of HDAC inhibitors have been identified including the following: 1) short-chain fatty acids [e.g., butyrates *(28,31)*]; 2) hydroxamic acids [e.g., trichostatin A (TSA) *(25,26)*, suberoylanilide hydroxamic acid (SAHA) *(34)*, and oxamflatin *(35)*]; 3) cyclic tetrapeptides containing a 2-amino-8-oxo-9,10-epoxy-decanoyl (AOE) moiety [e.g., trapoxin A) *(27)*]; 4) cyclic peptides not containing the AOE moiety [e.g., FR901228 and apicidin *(33,78)*]; and 5) benzamides [e.g., MS-27-275 *(32)*]. HDAC inhibitors invariably inhibit proliferation of transformed cells in culture, and a subset has been shown to inhibit tumor growth in animal models *(26,32,35–40)*. The butyrates represent the only class that is approved currently for use in the clinic. The butyrates are not ideal agents because of the high concentrations required (millimolar) to achieve inhibition of HDAC activity and multiple effects on other enzyme systems *(28,31)*. TSA, originally developed as an antifungal agent *(25,26,29)*, is a potent inhibitor of HDAC that is active at nanomolar concentrations. The finding that TSA-resistant cell lines have an altered HDAC is evidence that this enzyme is an important target for TSA. Oxamflatin *(35)*, a hydroxamic acid-based compound, and the benzamide MS-27-275 *(32)* inhibit HDAC activity at micromolar concentrations. Apicidin is a fungal metabolite that exhibits potent, broad-spectrum antiprotozoal activity and inhibits HDAC activity at nanomolar concentrations *(78)*. Depsipeptide (FR901228), isolated from *Chromobacterium violaceum (33)*, inhibits HDAC activity at micromolar concentrations. Trapoxin *(27)* and depudecin *(30)* irreversibly bind to HDAC and inhibit its activity at nanomolar and micromolar concentrations, respectively.

In our laboratory, a series of hydroxamic acid-based HPCs have been synthesized that inhibit HDACs at micromolar concentrations or lower *in vitro* and *in vivo (34,36,38,79)* (Fig. 4), and extensive structure–activity studies have been done with these compounds *(34,79)*. The essential characteristics of hydroxamic acid-based HPCs are a polar site, the hydroxamic group, a six-carbon hydrophobic methylene spacer, a second polar site, and a terminal hydrophobic group (Fig. 4). Substitution of the hydroxamic acid with a carboxylic acid or amide oxime group results in inactive compounds. Modification of the hydroxamic acid, such as introduction of a methyl group on an adjacent carbon or *N*-methylation, results in inactive compounds. The benzene ring in the hydrophobic moiety can be

Name	Structure	Opt. Conc.	% Diff.
SBHA		30 μM	90%
SAHA		2.5 μM	68%
CBHA		4.0 μM	73%
Pyroxamide		4.0 μM	51%

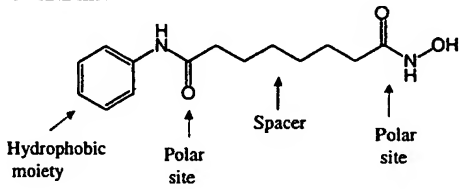


Fig. 4. Hydroxamic acid-based hybrid polar compounds. The optimal concentration to induce murine erythroleukemia cells to differentiate (% Diff) was determined from the percent of differentiated cells [detected as benzidine-stained cells (benzidine binds to the iron-containing heme of hemoglobin); for details of methods, *see (34)*]. SBHA = suberic bishydroxamic acid; SAHA = suberoylanilide hydroxamic acid; CBHA = *m*-carboxy-cinnamic acid bishydroxamic acid.

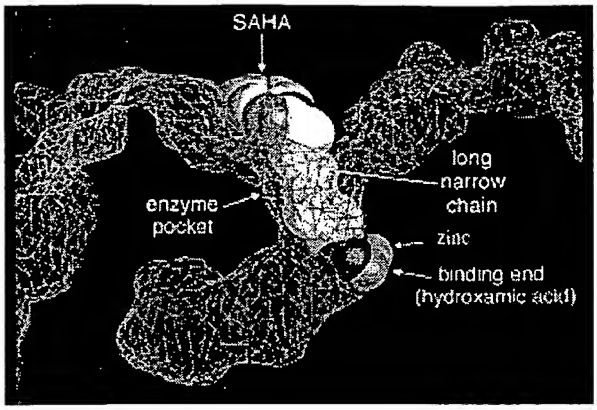


Fig. 5. SAHA (suberoylanilide hydroxamic acid) binds to the pocket of the catalytic site of a histone deacetylase-like protein, schematically represented by the netting. SAHA makes contact with residues at the rim, walls, and bottom of the pocket (enzyme pocket). The hydroxamic acid moiety of SAHA binds to the zinc at the bottom of the pocket *(80)*. (The figure is courtesy of Michael S. Finnin and Nikola P. Pavletich.)

modified in the meta and para positions without loss of activity; however, in general, larger substituents are associated with loss of activity. The optimal methylene spacer is six methylenes, five- and seven-carbons spacers being less active.

The structure of the catalytic core of HDACs has been determined by x-ray crystallography *(80)*. HDACs share an approximately 390-amino acid region of homology, referred to as the deacetylase core. Residues that form the active site are conserved across all HDACs. The deacetylase core identifies a gene superfamily that includes an HDAC homologue in the hyperthermophilic bacterium *Aquifex aeolicus* (termed "HDLP"), which was used for x-ray crystallography studies. There is a 35.2% base-pair identity between sequences of the catalytic core of the HDLP and of the mammalian HDAC1. HDLP deacetylates histones *in vitro*, its activity is inhibited by TSA and SAHA, but its specific activity is equal to about 7.5% of that of partially purified HDAC1. From x-ray crystallographic analyses of HDLP, an HDLP–TSA complex, and an HDLP–SAHA complex, the active catalytic site in the HDLP was shown to be formed by a tubular pocket, a zinc-binding site, and two asparagine–histidine charge-relay systems (Fig. 5). The hydroxamic acid moieties of TSA and SAHA bind to the zinc in the tubular pocket and the carbon-ring group projects out of the pocket on the surface of the protein.

Activity of HDAC Inhibitors *In Vitro*

The hydroxamic acid-based HPCs (e.g., *m*-carboxy-cinnamic acid bishydroxamic acid [CBHA], suberic bishydroxamic acid [SBHA], SAHA, and pyroxamide) (Fig. 4) inhibit partially purified HDAC1 and HDAC3 at concentrations of 0.01–1.0 μ*M* *(34)*. Furthermore, the optimal concentrations of various HPCs that induce murine erythroleukemia (MEL) cell differentiation as assayed by the proportion of cells that become benzidine positive (a stain for heme of hemoglobin) are correlated directly with the concentration required to inhibit the activity of partially purified HDAC1 or HDAC3 over a wide concentration range.

With the use of MEL cells and T24 human bladder carcinoma cells in culture, the effects of SAHA and related hydroxamic acid-based HPCs on the acetylation of histones have been examined *(34)*. SAHA, pyroxamide, SBHA, and CBHA (Fig. 4) cause accumulation of acetylated histones. Acetylated histone type-specific antibodies were used to show that, when cells were cultured with hydroxamic acid-based HPCs, the level of acetylation in histones H2A, H2B, H3, and H4 increased (Fig. 6). Increased histone acetylation could be detected as early as 1 hour after MEL or T24 cells were cultured with SAHA or other hydroxamic acid-based HPCs. The level of acetylated histones reached a maximum 6–12 hours after the addition of HPCs and remained elevated as long as the HPC was present *(34)*.

HDAC inhibitors can induce growth arrest, differentiation, and/or apoptotic cell death in a wide variety of cultured transformed cells, including neuroblastoma, melanoma, and leukemia cells, as well as cells from breast, prostate, lung, ovary, and colon cancers *(25–30,36,40,78,81)*. For example, SAHA induces terminal cell differentiation in several cell lines, including MEL, T24 human bladder carcinoma, and MCF-7 human breast adenocarcinoma. Differentiation was evaluated by parameters that included morphology, arrest in G_1 phase of the cell cycle, and developmental markers, such as hemoglobin in MEL cells, milk proteins in MCF-7 cells, and gelsolin in T24 cells. SAHA induces apoptotic death of human multiple myeloma cells (ARP-1), human prostate cell lines (LNCaP), and myelomonocytic leukemia cells (U937). CBHA induced apoptotic cell death of several human neuroblastoma cell lines, LAI-55n, KCN-69n, and SK-N-ER. Apoptosis was assayed by DNA fragmentation analysis and the deletion of a sub-G_1 (<2N ploidy) population by flow cytometry.

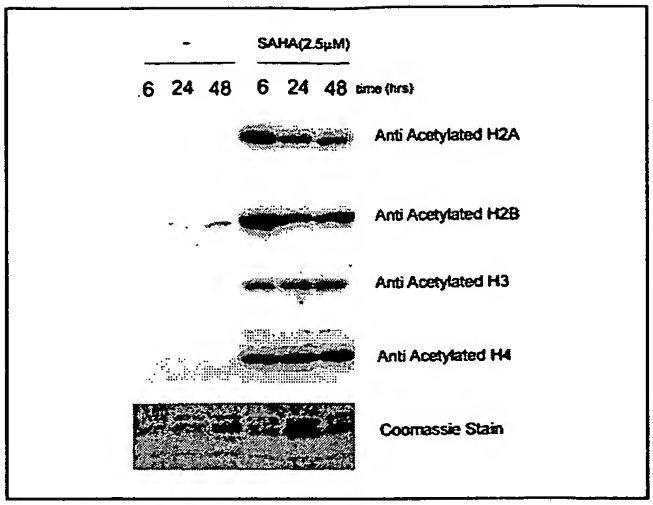


Fig. 6. Effect of SAHA (suberoylanilide hydroxamic acid) on histone acetylation in MEL cells. Cells were cultured without (–) or with 2.5 μ*M* SAHA for the times indicated. The acetylation of the histones was analyzed by use of antibodies specific for acetylated H2A, H2B, H3, and H4. The Coomassie-stained gel, **at the bottom,** indicates that the amount of protein loaded in each lane was similar [for details of methods, *see (36)*].

Van Lint et al. *(82)* have shown that the action of HDAC inhibitors on gene expression is selective. In cells cultured with TSA, the expression of only about 2% of expressed genes is changed (increased or decreased) twofold or more compared with untreated control cells. Our laboratory has obtained comparable results with transformed cells cultured with SAHA. The basis for the gene selectivity of SAHA or TSA is not known.

One gene most consistently induced by HDAC inhibitors is the cyclin-dependent kinase inhibitor $p21^{WAF1}$, which plays an important, if not determinant, role in the arrest of cell growth. Butyrate, TSA, depsipeptide, oxamflatin, MS-27-275, and the hydroxamic acid-based HPCs *(28,31,32,34,83)* induce $p21^{WAF1}$ transcription. The relation between SAHA-mediated histone hyperacetylation and increased $p21^{WAF1}$ gene expression has been studied in T24 human bladder carcinoma cells *(84)*. Increased transcription of the $p21^{WAF1}$ gene is associated with an increased level of acetylation on histones associated with the $p21^{WAF1}$ gene.

In Vivo Studies With HDAC Inhibitors

The butyrate analogue phenylbutyrate gave mixed results when tested as an HDAC inhibitor in animals and in a patient with APL. It was ineffective to moderately effective in inhibiting growth of solid tumors or leukemias, and that activity was observed only at relatively high doses *(28)*. A 13-year-old girl with relapsed APM who no longer responded to treatment with retinoic acid alone was treated with retinoic acid plus phenylbutyrate and had a complete clinical remission that was sustained for 7 months, during five treatment courses, before relapsing and becoming resistant to this treatment *(74)*. The acetylation of histones in her mononuclear blood cells was elevated during the period of administration of the phenylbutyrate. No remissions were induced in four other patients with APL *(75)*.

Several other HDAC inhibitors, including depsipeptide *(32)*, oxamflatin *(35)*, MS-27-275 *(32)*, and the hydroxamic acid-based HPCs *(37–39)*, inhibit tumor growth in animal models (Figs. 3 and 4). TSA did not inhibit the growth of a human melanoma xenograft in nude mice, but azeloic bishydroxamate did *(40)*. Treatment with HDAC inhibitors can increase the accumulation of acetylated histone in tumor tissue and/or normal tissues (e.g., spleen, bone marrow cells, and peripheral mononuclear cells). Thus, the level of acetylated histones is a useful intermediary marker of HDAC inhibitor activity.

Hydroxamic acid-based HPCs (Fig. 4) have been tested extensively in animal studies. One study *(37)* used rats with *N*-methylnitrosourea-induced mammary carcinomas. When these rats were fed SAHA (900 parts/million, continuously, beginning 7 days before the administration of *N*-methylnitrosourea), the incidence of mammary tumors was reduced by 40%, and the mean tumor volume was reduced by 78%—without side effects. Another study *(39)* used mice in which the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone induces lung tumors. When these mice were fed SAHA (900 parts/million, continuously, beginning 7 days before administration of the carcinogen to the end of the studies), the formation of lung tumors was substantially inhibited—also without toxic effects. A third rodent study *(38)* used nude mice bearing transplanted CWR22 androgen-dependent human prostate tumors. When these mice were given SAHA (25, 50, or 100 mg/kg per day) daily by intraperitoneal injection for 3 weeks, starting as soon as palpable tumors were detected, SAHA suppressed tumor growth at all three doses. With doses of 50 and 100 mg/kg per day, the mean tumor volume was reduced by 97%. Acetylation of histones H3 and H4 increased in the CWR22 tumor cells within 6 hours after SAHA was injected. Pyroxamide had similar effects on CWR22 tumor growth and the accumulation of acetylated histones (Fig. 4). When SAHA or pyroxamide was given at doses that markedly inhibited tumor growth, no toxicity, as evaluated by weight gain and histologic examination of multiple tissues at necropsy, was detected.

Conclusions

The studies summarized in this review indicate that the hydroxamic acid-based HPCs, in particular, SAHA and pyroxamide—are potent inhibitors of HDAC *in vitro* and *in vivo* and induce growth arrest, differentiation, or apoptotic cell death of transformed cells. We suggest that inhibition of HDAC activity leads to relaxation of the structure of chromatin associated with a specific set of programmed genes. The relaxed chromatin structure allows these genes to be expressed, which, in turn, arrests tumor cell growth. SAHA and pyroxamide are lead compounds among the family of hydroxamic acid-based HPCs and are currently in phase I clinical trials.

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NOTES

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